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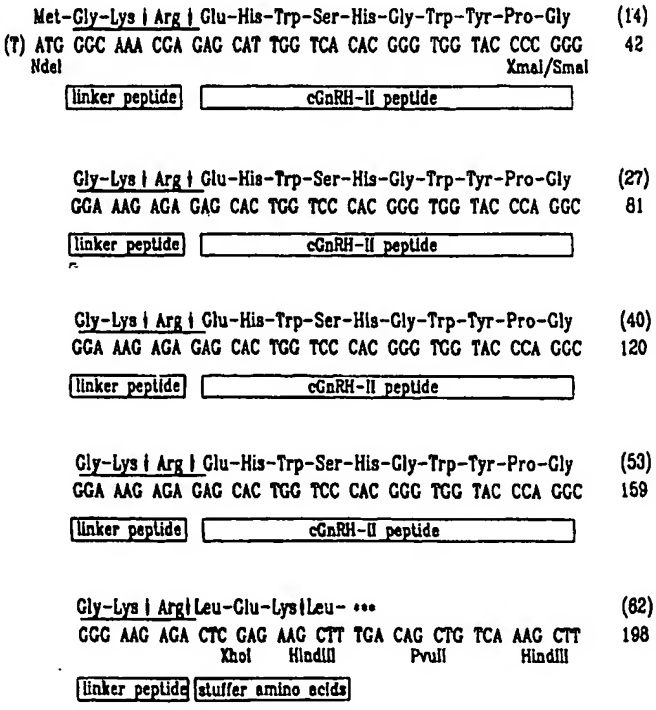
(57) Abstract

A biologically active peptide is prepared by: a) preparing an expression vector comprising a DNA cassette containing two or more tandem repeating units of a nucleotide sequence encoding a biologically active peptide and a linker peptide attached thereto, the linker peptide being cleavable by a protease or a chemical agent; b) transforming a microorganism with the expression vector; c) culturing the transformed microorganism to produce a multimeric peptide expressed by the DNA cassette; d) recovering and digesting the multimeric peptide with the protease or the chemical agent to obtain the biologically active peptide or an analog thereof carrying one or more amino acid residues originating from the linker peptide.



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# DNA CASSETTE ENCODING A MULTIMER OF A BIOLOGICALLY ACTIVE PEPTIDE AND A CLEAVABLE LINKER ATTACHED THERETO AND PROCESS FOR PREPARING THE BIOLOGICALLY ACTIVE PEPTIDE

## FIELD OF THE INVENTION

The present invention relates to a DNA cassette comprising two or more tandem repeating units of a nucleotide sequence encoding a biologically active peptide and a cleavable linker peptide attached thereto, an expression vector containing the DNA cassette, a microorganism transformed by the expression vector, and a process for preparing the biologically active peptide using the transformed microorganism.

## BACKGROUND OF THE INVENTION

Many biologically active peptides such as enzymes and hormones have been produced by chemical synthesis or by in vivo synthesis using genetically engineered host microorganisms.

The chemical synthesis method has been employed mainly for the production of very short peptides comprised of 2 to 10 amino acid residues. This chemical method is, however, ineffective and uneconomical when applied to the production of peptides having more than 10 amino acid residues.

The method of producing biologically active peptides in genetically engineered host microorganisms, on the other hand, has been widely used for the production of long polypeptides having 100 to 400 amino acid residues, e.g., growth hormones, insulin and restriction enzymes. However, this method is not suitable for preparing shorter peptides with less than 100 amino acid residues because of its inherent low productivity.

Accordingly, there has existed a need to develop an economical process for preparing short biologically active

peptides comprising 10 to 100 amino acid residues.

## SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide a novel DNA cassette containing multiple copies of a nucleotide sequence encoding a short peptide, an expression vector comprising the DNA cassette, a microorganism transformed by the vector, and a process for preparing the peptide using the transformed microorganism.

In accordance with one aspect of the present invention, there is provided a DNA cassette which comprises two or more tandem repeating units of a nucleotide sequence encoding a biologically active peptide and a linker peptide attached thereto, said linker peptide being cleavable by a protease or a chemical agent.

## BRIEF DESCRIPTION OF THE DRAWINGS

The above objects and features of the present invention will become apparent from the following description of preferred embodiments taken in conjunction with the accompanying drawings, in which:

Fig. 1 shows the DNA fragment obtained by annealing oligonucleotides 1 and 2;

Fig. 2 shows the procedure of designing the basic DNA unit.

Fig. 3 depicts the left adaptor obtained by annealing oligonucleotides 3 and 4;

Fig. 4 represents the right adaptor obtained by annealing oligonucleotides 5 and 6;

Fig. 5 reproduces the electrophoresis result of the 174 bp DNA cassette;

Fig. 6 presents the DNA fragment obtained by annealing oligonucleotides 7;

Fig. 7 discloses the synthetic DNA fragment obtained by

annealing oligonucleotides 8 and 9;

Fig. 8 describes the procedure for constructing vector pRSET-B-cGnRH-II'-4R*;

Fig. 9 provides the nucleotide sequence of the DNA cassette contained in vector pRSET-B-cGnRH-II'-4R*;

Fig. 10 offers the SDS-PAGE result of the 7.3 kDa protein expressed by vector pRSET-B-cGnRH-II'-4R*; and

Fig. 11 discloses the electrophoresis result of the peptides obtained by digesting the 7.3 kDa protein with trypsin.

## DETAILED DESCRIPTION OF THE INVENTION

A DNA cassette of the present invention comprises two or more tandem repeating units of a nucleotide sequence encoding a biologically active peptide and a linker peptide attached thereto, said linker peptide being cleavable by a protease or a chemical agent.

The biologically active peptide which may be used in the present invention includes any biologically active peptide comprised of 2 to 200 amino acids, preferably 10 to 100 amino acids. Exemplary peptides include adrenocorticotropic hormone, angiotensin, atrial natriuretic peptide, bradykinin, chemotactic hormone, dynorphin, endorphin, enkephalin, fibronectin, ß-lipotropin fragments, gastrointestinal peptide, growth hormone releasing peptide, luteinizing hormone releasing hormone such as gonadotropin releasing hormone, melanocyte stimulating hormone, neurotensin, opioid peptide, oxytocin, vasopressin, vasotocin, parathyroid hormone, somatostatin, substance P and antigenic peptide.

The linker peptide may have an amino acid residue or an amino acid sequence which is specifically recognized and digested by a protease or a chemical agent, thereby providing one or more cleavage sites. Many protease- or chemical agent-digestible amino acid links and amino acid

sequences are well known in the art of protein biochemistry; and representative examples thereof are shown in Table I.

Table I

| Protease or Chemical Agent | | Digestible Amino Acid or Amino Acid Sequence |
|---|---|---|
| Protease | Trypsin | Lys↓ or Arg↓ |
| | Asparaginyl-endopeptidase | Asn↓ |
| | Arginyl-endopeptidase | Arg↓ |
| | Achromobacter Protease I | Lys↓ |
| | Staphylococcus aureus V8 Protease | Glu↓ |
| | rTEV Protease | Glu-Asn-Leu-Tyr-Phe-Gln↓-Gly |
| | Factor Xa Protease | Ile-Glu-Gly-Arg↓ |
| Chemical Agent | Cyanogen bromide | Met↓ |

* ↓ represents a peptide bond digested by a protease or chemical agent.

Using one or more digestible amino acid links and amino acid sequences as set forth above, various linker peptides may be designed as shown in Table II.

Table II

| Protease or Chemical Agent | Linker Peptide |
|---|---|
| Trypsin | Lys<br>Arg<br>Lys-Arg<br>Arg-Lys<br>Lys-$(X)_n$<br>$(X)_n$-Lys<br>Arg-$(X)_n$<br>$(X)_n$-Arg<br>$(X)_n$-Arg-Lys<br>Lys-Arg-$(X)_n$<br>Arg-Lys-$(X)_n$<br>$(X)_n$-Lys-Arg-$(X)_n$<br>$(X)_n$-Arg-Lys-$(X)_n$<br>$(X)_n$-Arg-$(X)_n$-Lys<br>$(X)_n$-Lys-$(X)_n$-Arg<br>$(X)_n$-Lys-$(X)_n$-Arg-$(X)_n$<br>$(X)_n$-Arg-$(X)_n$-Lys-$(X)_n$ |
| Factor Xa Protease | Ile-Glu-Gly-Arg<br>Ile-Glu-Gly-Arg-$(X)_n$<br>$(X)_n$-Ile-Glu-Gly-Arg<br>$(X)_n$-Ile-Glu-Gly-Arg-$(X)_n$ |
| Cyanogen Bromide | Met<br>$(X)_n$-Met<br>Met-$(X)_n$<br>$(X)_n$-Met-$(X)_n$ |

* X is Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr or Val; and n is an integral number.

A linker peptide attached to a biologically active peptide is used as a basic repeating unit to prepare a tandem repetitive multimeric peptide which yields multiple copies of the biologically active peptide when digested by a protease or a chemical agent.

A biologically active peptide having a digestible C-terminal amino acid residue, e.g., lysine, arginine, asparagine, glutamine and methionine, may also be used in the present invention as a basic repeating unit.

A nucleotide sequence encoding a basic repeating unit may be tandemly linked together to form a DNA cassette encoding a multimeric peptide.

The DNA cassette encoding the multimeric peptide may be

obtained by ligating a basic DNA unit encoding the above mentioned basic repeating unit in accordance with a conventional ligation method(Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, USA(1989)).

The basic DNA unit may be any of 5'-cohesive ended form, 3'-cohesive ended form and blunt-ended form. Among those, the 5'-cohesive ended form and 3'-cohesive ended form are preferred because of their ability to undergo a directional ligation to generate a DNA cassette having directional tandem repeats thereof. Particularly preferred is a DNA fragment having a double stranded DNA region encoding a target peptide and complementary single strand DNA sequences of a linker peptide protruding from 5'- or 3'-ends thereof. Such basic DNA unit may be obtained by chemically synthesizing a pair of complementary oligonucleotides encoding the basic repeating unit and annealing the oligonucleotides in accordance with a conventional annealing method(Sambrook et al., vide supra).

The nucleotide sequence of a basic DNA unit encoding a basic repeating unit may be deduced from the amino acid sequences of the target peptide and the linker peptide contained in the basic repeating unit based on the standard genetic code.

The nucleotide sequence encoding a basic repeating unit may be further modified without changing the amino acid sequence so as to minimize the free energy of the secondary structure of RNA transcribed therefrom in case of N-terminal basic repeating unit, thereby increasing the expression efficiency thereof; or to lower the nucleotide homology between repeating nucleotide sequences; or to provide one or more preferred codons.

Therefore, the basic DNA unit which may be used in the ligation reaction has a nucleotide sequence encoding a repeating unit and various modified forms thereof.

The DNA cassette of the present invention may be

obtained by ligating in tandem a suitable number of basic DNA units encoding the basic repeating unit, the number ranging from 2 to 20, preferably 2 to 10. Such cassette encodes a multimeric peptide containing multiple copies of the basic repeating unit in tandem; and it may further contain an additional nucleotide sequence encoding a stuffer amino acid in the linker peptide region to modify the charge or hydrophobicity of the multimeric peptide.

The vector of the present invention, which is capable of expressing a multimeric peptide, may be obtained by inserting the DNA cassette containing a nucleotide sequence encoding a multimeric peptide in an expression vector according to a conventional cloning method(Sambrook et al., vide supra). In this process, suitable adaptors may be attached to the DNA cassette in order to facilitate the cloning thereof before the insertion.

The expression vector thus obtained may be introduced into a host microorganism such as E. coli, in accordance with a conventional transformation method(Sambrook et al., the supra).

A biologically active peptide or an analog thereof may be efficiently prepared by (a) culturing the transformed microorganism to produce a multimeric peptide expressed by the DNA cassette; (b) recovering the multimeric peptide from the culture; and (c) digesting the multimeric peptide with a protease or a chemical agent to obtain the biologically active peptide or the analog thereof, carrying one or more amino acid residues originating from the linker peptide.

The transformed host microorganism may be cultured in accordance with a conventional method(Sambrook et al., the supra) to express the multimeric peptide. The expression may be induced using an appropriate inducer, e.g., isopropyl-1-thio-ß-D-galactoside.

The microorganism cell culture may be centrifuged or filtered to harvest microorganism cells. The cells are then lysed; and the multimeric peptide may be isolated therefrom

by a conventional method(Methods in Enzymology, volume 182, Edited by Murray D. Deutscher, Academic press, Inc., New York, USA), e.g., ion exchange chromatography or gel filtration column chromatography, preferably anion exchange fast protein liquid chromatography, and, more preferably, Mono-Q anion exchange fast liquid chromatography(Pharmacia LKB, Sweden).

The purified multimeric peptide may be digested with an appropriate protease or chemical agent in accordance with a conventional digestion method(Methods in Enzymology, vide supra) to obtain a biologically active peptide, or an analog having one or more linker peptide-derived amino acids attached to N- or C-terminus thereof.

The following Preparation Examples and Examples are intended to further illustrate the present invention without limiting its scope.

Preparation Example 1: Preparation of Basic DNA Unit

A trypsin-digestible linker peptide represented by following amino acid sequence(SEQ ID NO: 1) was designed.

Gly Lys Arg

Based on the amino acid sequences of chicken gonadotropin releasing hormone II(designated cGnRH-II; Miyamoto et al., Proc. Natl. Acad. Sci. USA, 81, 3874-3878 (1984)) and the above linker peptide, a basic peptide unit represented by following amino acid sequence was designed. This unit, designated cGnRH-II', has the structure of linker peptide-cGnRH-II-linker peptide.

cGnRH-II(SEQ ID NO: 2):

Glu His Trp Ser His Gly Trp Tyr Pro Gly

5 10

cGnRH-II' (linker peptide-cGnRH-II-linker peptide) (SEQ ID NO: 3):

Gly Lys Arg Glu His Trp Ser His Gly Trp Tyr Pro Gly Gly Lys
5 10 15
Arg

Using the standard genetic codes, a DNA fragment having the following nucleotide sequence(SEQ ID NO: 4) was deduced to match cGnRH-II'.

5'-GGGAAGAGAGAGCACTGGTCCCACGGGTGGTACCCAGGCGGGAAGAGA-3'

To obtain a basic DNA unit encoding cGnRH-II', the following pair of complementary oligonucleotides were synthesized and annealed to obtain a basic DNA unit shown in Fig. 1 which consists of a double stranded(ds) region encoding cGnRH-II and two single stranded(ss) regions protruding from both 3' ends thereof encoding the linker peptide, the two single strands being complementary to each other.

sense strand oligonucleotide 1 (SEQ ID NO: 5):
5'-GAGCACTGGTCCCACGGGTGGTACCCAGGCGGGAAGAGA-3'
antisense strand oligonucleotide 2 (SEQ ID NO: 6):
5'-GCCTGGTGACCACCCGTGGGACCAGTGCTCTCTCTTCCC-3'

Fig. 2 shows the procedure of designing the basic DNA unit.

This basic DNA unit was employed as a monomer in the preparation of a DNA cassette encoding a multimeric peptide composed of repeating units of (cGnRH-II-linker peptide).

Preparation Example 2: Preparation of Left Adaptor

For preparing a left adaptor useful for cloning the DNA fragment obtained in Preparation Example 1, the following

pair of complementary oligonucleotides were synthesized and annealed to obtain a left adaptor shown in Fig. 3 which consists of a 5'-protruding ss region having EcoRI site, a ds region having BamHI and XmaI/SmaI sites, and a 3'-protruding ss region having a nucleotide sequence encoding the linker peptide.

oligonucleotide 3 (SEQ ID NO: 8):
5'-AATTCAAGGATCCCCCGGGGGGAAGAGA-3'
oligonucleotide 4 (SEQ ID NO: 9):
5'-CCCGGGGGATTCTTG-3'

Preparation Example 3: Preparation of Right Adaptor

The procedure of Preparation Example 2 was repeated except for using the following pair of complementary oligonucleotides, to obtain a right adaptor shown in Fig. 4 which consists of a 3'-protruding ss region having a nucleotide sequence complementary to that the encoding linker peptide, a ds region having XhoI and HindIII sites, and a 5'-protruding ss region having SalI site.

oligonucleotide 5 (SEQ ID NO: 10):
5'-CTCGAGAAGCTTACG-3'
oligonucleotide 6 (SEQ ID NO: 11):
5'-TCGACGTAAGCTTCTCGAGTCTCTTCCC-3'

Example 1: Construction of DNA Cassette

The basic DNA unit obtained in Preparation Example 1 was self-ligated at 16 °C for 2 hours to obtain a DNA cassette comprised of tandem repeats of the DNA unit.

The DNA cassette was ligated with the left and right adaptors obtained in Preparation Examples 2 and 3, respectively, using T4 DNA ligase at 16 °C for 2 hours to obtain a DNA cassette. The DNA cassette was inserted in the

EcoRI/SalI sites of plasmid pUC19(Yanish-Peron, C. et al., Gene, 33, 103-119(1985)). E. coli JM109 was transformed with the plasmid.

The transformant E. coli was cultured in LB medium(bacto-yeast extract 5 g, bacto-tryptone 10 g, NaCl 5 g and agar 15 g(pH7.2) per ℓ) containing 50 μg/ml ampicillin at 37 °C overnight. Plasmid DNA was purified from the culture and cleaved with EcoRI and SalI. DNA fragments thus obtained were subjected to electrophoresis on 3% metaphor agarose gel(FMC Co., USA).

Fig. 5 reproduces the result of electrophoresis wherein lane 1 represents a 47 bp DNA fragment which is obtained by ligating the left and right adaptors; lane 2, a 86 bp DNA cassette which results from the ligation of the left adaptor, the basic DNA unit and the right adaptor; lane 3, a 174 bp DNA cassette which is the product of ligating the left adaptor, three basic DNA units and the right adaptor; and lane 4, a 1kb ladder DNA molecular size markers.

The plasmid containing 174 bp DNA cassette was designated pUC19-cGnRH-II'-3R.

Example 2: Construction of Expression Vector containing DNA Cassette

(Step 1) Insertion of a DNA cassette into an expression vector

Plasmid pUC19-cGnRH-II'-3R obtained in Example 1 was cleaved with BamHI and HindIII and the DNA cassette thus obtained was inserted in the BamHI/HindIII sites of expression vector pRSET-B(Invitrogen Co., USA) to obtain vector pRSET-B-cGnRH-II'-3R.

(Step 2) Addition of a termination codon to a DNA cassette

To add a termination codon to the DNA cassette, a DNA

fragment containing a termination codon, which is shown in Fig. 6, was prepared by annealing the following self-complementary oligonucleotide 7 (SEQ ID NO: 12), which provides a termination codon(TGA), an NdeI site and an XmaI site, and the DNA fragment thus obtained was inserted in the HindIII site of vector pRSET-B-cGnRH-II'-3R obtained in Step 1, to obtain vector pRSET-B-cGnRH-II'-3R*.

5'-AGCTTTGACAGCTGTCAA-3'

(Step 3) Addition of a modified basic DNA unit

To increase the expression efficiency of the DNA cassette, the following complementary oligonucleotides were synthesized and annealed to obtain a synthetic DNA fragment shown in Fig. 7, which encodes methionine-linker peptide-cGnRH-II with a lower ΔG value for RNA secondary structure(computer program DNASIS™, Hitachi, Japan), and using the DNA fragment thus obtained the NdeI/XmaI fragment of vector pRSET-B-cGnRH-II'-3R* obtained in Step 2 was replaced, to obtain vector pRSET-B-cGnRH-II'-4R*.

oligonucleotide 8 (SEQ ID NO: 13):
5'-TATGGGCAAACGAGAGCATTGGTCACACGGGTGGTACC-3'
oligonucleotide 9 (SEQ ID NO: 14):
5'-CCCGGGTACCACCCGTGTGACCAATGCTCTCGTTTGCCCAT-3'

Fig. 8 describes the procedure for constructing vector pRSET-B-cGnRH-II'-4R* wherein C shows a basic DNA unit; ΔC, modified basic DNA unit; L, a left adaptor; R, a right adaptor; and S, a termination codon.

The nucleotide sequence of the DNA cassette contained in vector pRSET-B-cGnRH-II'-4R* was determined using the dideoxy-mediated chain-termination method(Sanger, F. et al., Proc. Natl. Acad. Sci. USA, 74, 5463-5467 (1977)). The resulting nucleotide sequence(SEQ ID NO: 15) of the DNA

cassette is shown below and details of the sequence are represented in Fig. 9 wherein ↓ shows the trypsin-digestible site; and ***, the termination codon.

5'-ATGGGCAAACGAGAGCATTGGTCACACGGGTGGTACCCCGGGGGGAAGAGAGAGC
ACTGGTCCCACGGGTGGTACCCAGGCGGGAAGAGAGAGCACTGGTCCCACGGGTGGTA
CCCAGGCGGGAAGAGAGAGCACTGGTCCCACGGGTGGTACCCAGGCGGGAAGAGACTC
GAGAAGCTTTGACAGCTGTCAAAGCTT-3'

As can be seen in above nucleotide sequence and Fig. 9, the DNA cassette of vector pRSET-B-cGnRH-II'-4R* consists of 183 bp nucleotides which contains four repeats of the nucleotide sequence encoding the repeating unit of (cGnRH-II-linker peptide) arranged in an open reading frame and a termination codon.

E. coli TOP10F'(Invitrogen Co., USA) was transformed with vector pRSET-B-cGnRH-II'-4R* to obtain transformant E. coli cGnRH-II'-4R*.

Example 3: Expression of the DNA cassette

Transformant E. coli cGnRH-II'-4R* obtained in Example 2 was cultured in SOB medium(tryptone 20 g, yeast extract 5.0 g, NaCl 0.5 g and KCl 1.86 mg per 1 ℓ) containing 50 μg/ml ampicillin at 37 °C overnight. The resulting culture was diluted 200 fold with SOB medium and then incubated at 37 °C. When the optical density at 600 nm reached 0.3, isopropyl-1-thio-ß-D-galactoside was added thereto to a concentration of 1 mM. 5 pfu/cell of M13/T7 phage(Invitrogen Co., USA) was added thereto, and the phage infected culture was incubated for 5 hours to express the DNA cassette.

1 ml of the culture was centrifuged at 6,000 rpm for 5 min to obtain a pellet and the pellet was suspended in 200 μl of buffer(0.05M Tris-HCl, pH 6.8, 0.1M DTT, 2% SDS, 1% glycerol, and 0.1% bromophenol blue). The suspension was

heated at 90 °C for 5 min. and 10 μl thereof was subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis(SDS-PAGE) on 16% gel(Laemmli, U.K., Nature, 227, 680-685 (1970)).

Fig. 10 reproduces the result of SDS-PAGE, wherein lane 1 shows protein size markers; lane 2, proteins expressed in E. coli transformed with vector pRSET-B-cGnRH-II'-3R*; lane 3, proteins expressed in E. coli transformed with vector pRSET-B-cGnRH-II'-4R*. As can be seen in Fig. 10, vector pRSET-B-cGnRH-II'-3R* expresses a low level of 9.2 kDa protein while vector pRSET-B-cGnRH-II'-4R* expresses a protein having a size of 8.0 kDa which is close to 7.3 kDa estimated for the DNA cassette shown in Fig. 9.

Example 4: Production of cGnRH-II analog

The culture obtained in Example 3 was centrifuged at 2,000 x g for 20 min. at 4 °C to harvest E. coli cells. The E. coli cells were washed twice with cold 20mM Tris-HCl buffer(pH 8.0) and suspended in 1/10 volume of the original culture. The resulting suspension was subjected to fast protein liquid chromatography. Proteins were adsorbed on a Mono Q 5/5 anion exchange column(Pharmacia LKB, Sweden) pre-equilibrated with 100% buffer A(20 mM Tris-HCl(pH 8.0)) and eluted at a flow rate of 1 ml/min with a linear gradient generated using 100% buffer A and 100% buffer B(20mM Tris-HCl at pH 8.0 containing 1.0M NaCl). Combined protein fractions were desalted by dialyzing against 20 mM Tris-HCl(pH 8.0) and lyophilized. The lyophilized powder was resuspended in 20 mM Tris-HCl(pH 8.0) and then digested with trypsin at room temperature for 3 hours.

The digested protein was subjected to electrophoresis on Tricine gel having a concentration gradient from 10 to 20%(Novex, USA). The results are shown in Fig. 11, wherein lane 1 shows a 7.3 kDa protein; lanes 5, the peptide obtained by digesting the 7.3 kDa protein with trypsin; and

lane 6, trypsin. As can be seen in Fig. 11, the 7.3 kDa protein is a tandem repetitive multimer of the 1.4 kDa monomer peptide.

In order to examine the amino acid sequence of 1.4 kDa peptide, the 1.4 kDa peptide was transferred to a polyvinylidene difluoride membrane(Matsudaria, 1987) and the amino acid sequence was determined with a protein sequencer(Applied Biosystem, model 476A, USA) in a pulse-liquid mode. The resulting sequence(SEQ ID NO: 16) of the 1.4 kDa peptide is shown below.

Glu His Trp Ser His Gly Trp Tyr Pro Gly Gly Lys
5 10

As can be seen from the above amino acid sequence, the 1.4 kDa peptide is a cGnRH-II analog consisting of cGnRH-II having the linker-derived Gly-Lys sequence attached to the C-terminus.

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes may be made to the invention by those skilled in the art which also fall within the scope of the invention as defined by the appended claims.

What is claimed is:

1. A DNA cassette which comprises two or more tandem repeating units of a nucleotide sequence encoding a biologically active peptide and a linker peptide attached thereto, the linker peptide being cleavable by a protease or a chemical agent.

2. The DNA cassette of claim 1, wherein the biologically active peptide is comprised of 2 to 200 amino acid residues.

3. The DNA cassette of claim 2, wherein the biologically active peptide is selected from the group consisting of adrenocorticotropic hormone, angiotensin, atrial natriuretic peptide, bradykinin, chemotactic hormone, dynorphin, endorphin, enkephalin, fibronectin, ß-lipotropin fragments, gastrointestinal peptide, growth hormone releasing peptide, luteinizing hormone releasing hormone, melanocyte stimulating hormone, neurotensin, opioid peptide, oxytocin, vasopressin, vasotocin, parathyroid hormone, somatostatin, substance P and antigenic peptide.

4. The DNA cassette of claim 3, wherein the biologically active peptide is chicken gonadotropin releasing hormone II.

5. The DNA cassette of claim 4, which has a following nucleotide sequence(SEQ ID NO: 15):

5'-ATGGGCAAACGAGAGCATTGGTCACACGGGTGGTACCCCGGGGGGAAGAGAGAGC
ACTGGTCCCACGGGTGGTACCCAGGCGGGAAGAGAGAGCACTGGTCCCACGGGTGGTA
CCCAGGCGGGAAGAGAGAGCACTGGTCCCACGGGTGGTACCCAGGCGGGAAGAGACTC
GAGAAGCTTTGACAGCTGTCAAAGCTT-3'.

6. The DNA cassette of claim 1, wherein the linker

peptide cleavable by a protease has an amino acid link or an amino acid sequence selected from the group consisting of:

(i) lysine and arginine, which are cleavable by trypsin;

(ii) asparagine, cleavable by asparaginylendopeptidase;

(iii) arginine, cleavable by arginylendopeptidase;

(iv) lysine, cleavable by Achromobacter protease I;

(v) glutamine, cleavable by Staphylococcus aureus V8 protease;

(vi) Glu-Asn-Leu-Tyr-Phe-Gln-Gly, cleavable by tTEV protease; and

(vii) Ile-Glu-Gly-Arg, cleavable by factor Xa.

7. The DNA cassette of claim 6, wherein the linker peptide is Gly-Lys-Arg.

8. The DNA cassette of claim 1, wherein the linker peptide cleavable by a chemical agent has an amino acid sequence containing methionine which is cleavable by cyanogen bromide.

9. A process for preparing a DNA cassette which comprises ligating a basic DNA unit having a 5'-cohesive ended form or 3'-cohesive ended form, the basic DNA unit composed of a double stranded DNA region encoding a biologically active peptide and two complementary single stranded DNA regions protruding from 5'- or 3'-ends thereof encoding a linker peptide cleavable by a protease or a chemical agent.

10. A DNA cassette which comprises two or more tandem repeating units of a nucleotide sequence encoding a biologically active peptide having a C-terminal amino acid residue which is cleavable by a protease or a chemical agent.

11. The DNA cassette of claim 10, wherein the biologically active peptide is comprised of 2 to 200 amino acid residues.

12. The DNA cassette of claim 10, wherein the C-terminal amino acid residue is selected from the group consisting of:

(i) lysine and arginine, which are cleavable by trypsin;

(ii) asparagine, cleavable by asparaginylendopeptidase;

(iii) arginine, cleavable by arginylendopeptidase;

(iv) lysine, cleavable by Achromobacter protease I; and

(v) glutamine, cleavable by Staphylococcus aureus V8 protease.

13. The DNA cassette of claim 10, wherein the C-terminal amino acid residue is methionine which is cleavable by cyanogen bromide.

14. An expression vector comprising the DNA cassette of any of claims 1 to 8 and 10 to 13.

15. The expression vector of claim 14, which is pRSET-B-cGnRH-II'-4R*.

16. A microorganism transformed with the expression vector of claim 14.

17. The microorganism of claim 16, which is E. coli transformed with pRSET-B-cGnRH-II'-4R*.

18. A process for preparing a biologically active peptide or an analog thereof which comprises the steps of:
(a) preparing an expression vector comprising a DNA cassette containing two or more tandem repeating units of a

nucleotide sequence encoding a biologically active peptide and a linker peptide attached thereto, the linker peptide being cleavable by a protease or a chemical agent;

(b) transforming a microorganism with the expression vector;

(c) culturing the transformed microorganism to produce a multimeric peptide expressed by the DNA cassette; and

(d) recovering and digesting the multimeric peptide with the protease or the chemical agent to obtain the biologically active peptide or an analog thereof carrying one or more amino acid residues originating from the linker peptide.

19. A process for preparing a biologically active peptide which comprises the steps of:

(a) preparing an expression vector comprising a DNA cassette containing two or more tandem repeating units of a nucleotide sequence encoding a biologically active peptide having a C-terminal amino acid residue which is cleavable by a protease or a chemical agent;

(b) transforming a microorganism with the expression vector;

(c) culturing the transformed microorganism to produce a multimeric peptide expressed by the DNA cassette; and

(d) recovering and digesting the multimeric peptide with the protease or the chemical agent to obtain the biologically active peptide.

*FIG. 1*

5'-GAG CAC TGG TCC CAC GGG TGG TAC CCA GGC GGG AAG AGA-3'
3'-CCC TTC TCT CTC GTG ACC AGG GTG CCC ACC ATG GGT CCG-5'

# FIG.2

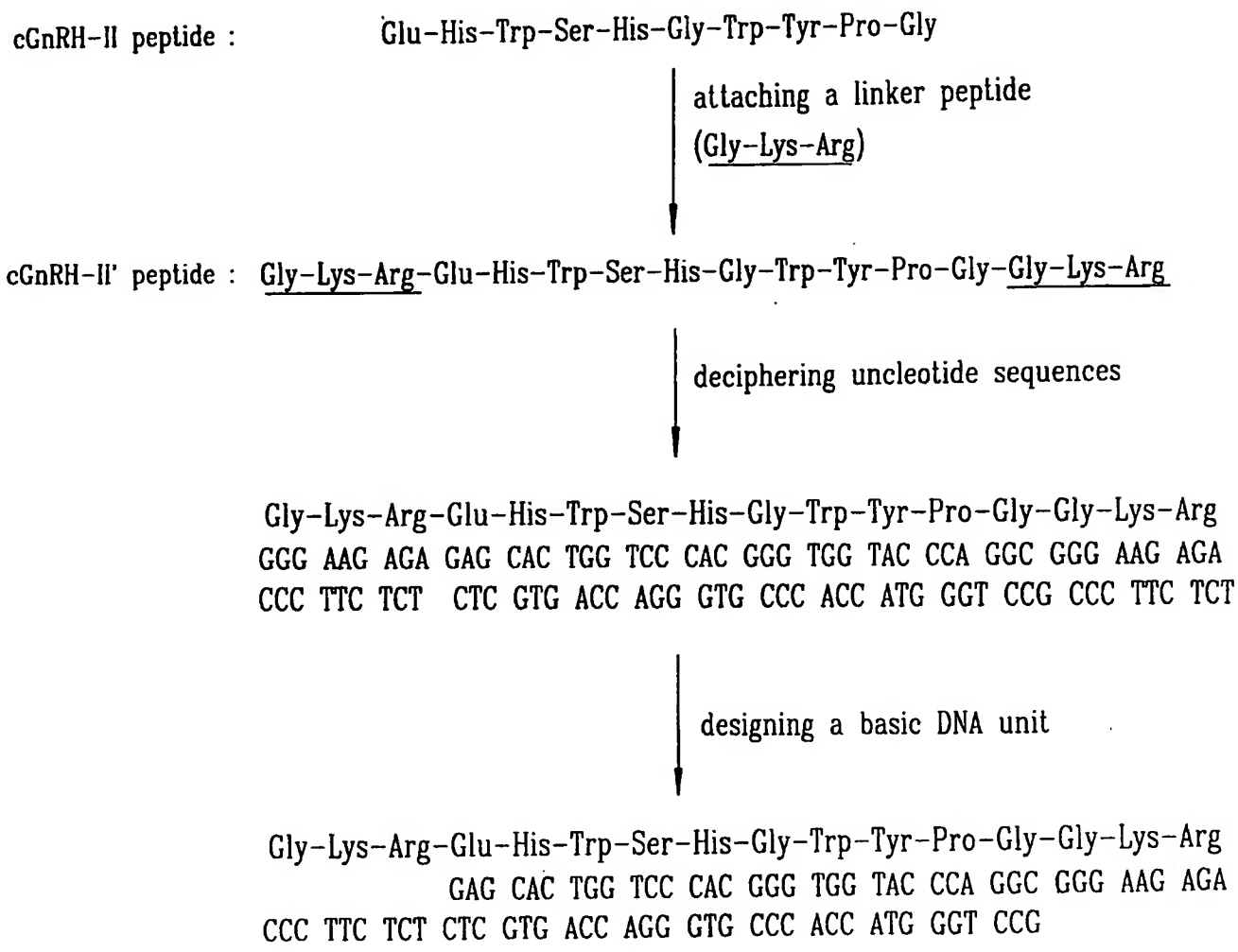


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*FIG.3*

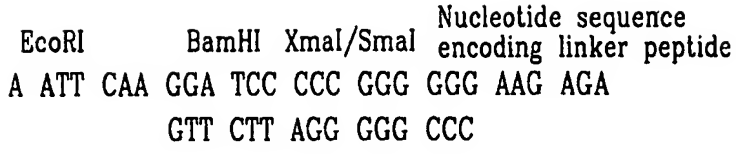
EcoRI
BamHI
XmaI/SmaI
Nucleotide sequence encoding linker peptide
A ATT CAA GGA TCC CCC GGG GGG AAG AGA
GTT CTT AGG GGG CCC

*FIG.4*

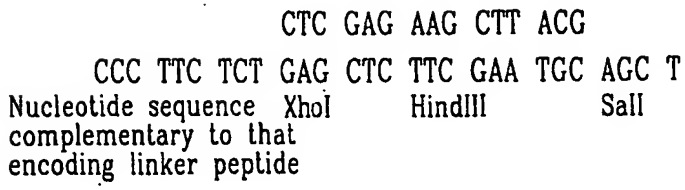
CTC GAG AAG CTT ACG
CCC TTC TCT GAG CTC TTC GAA TGC AGC T
Nucleotide sequence complementary to that encoding linker peptide
XhoI
HindIII
SalI

FIG.5

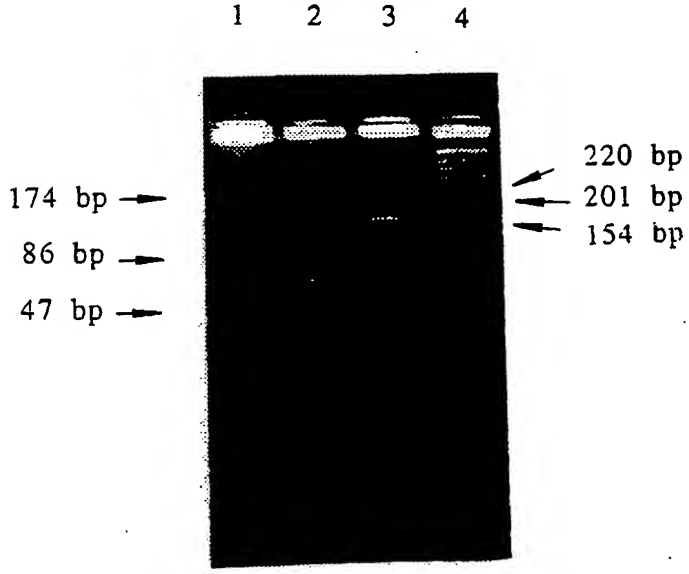


FIG.6

5'-AG CTT TGA CAG CTG TCA A-3'
3'-A ACT GTC GAC AGT TTC GA-5'

FIG.7

5'-T ATG GGC AAA CGA GAG CAT TGG TCA CAC GGG TGG TAC C -3'
3'-TAC CCG TTT GTC CTC GTA ACC AGT GTG CCC ACC ATG GGC CC-5'

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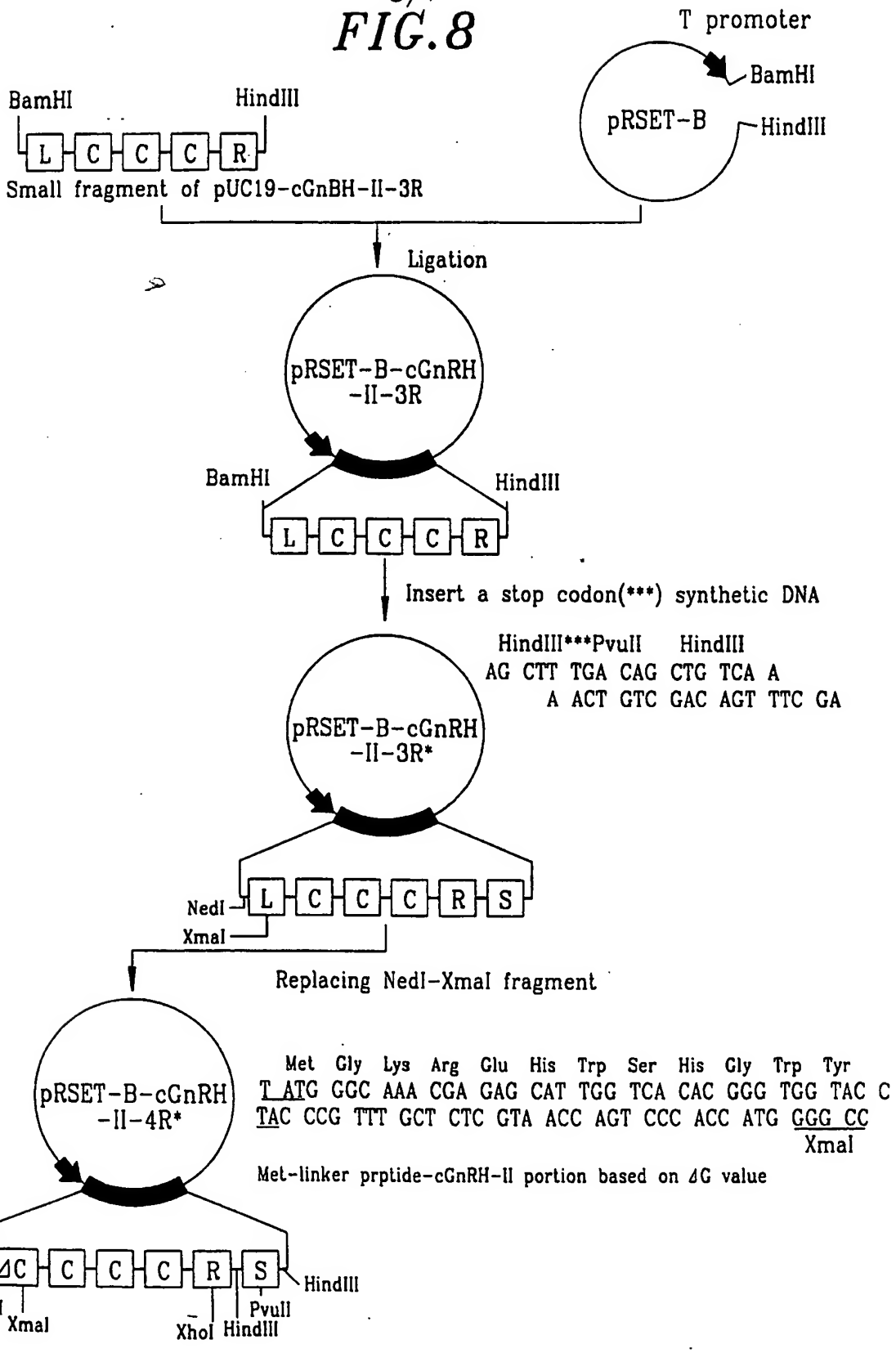
FIG.8


T promoter
BamHI
pRSET-B
HindIII
BamHI
HindIII
L C C C R
Small fragment of pUC19-cGnBH-II-3R
Ligation
pRSET-B-cGnRH -II-3R
BamHI
HindIII
L C C C R
Insert a stop codon(***) synthetic DNA
HindIII***PvuII HindIII
AG CTT TGA CAG CTG TCA A
A ACT GTC GAC AGT TTC GA
pRSET-B-cGnRH -II-3R*
NedI
XmaI
L C C C R S
Replacing NedI-XmaI fragment
pRSET-B-cGnRH -II-4R*
Met Gly Lys Arg Glu His Trp Ser His Gly Trp Tyr
T ATG GGC AAA CGA GAG CAT TGG TCA CAC GGG TGG TAC C
TAC CCG TTT GCT CTC GTA ACC AGT CCC ACC ATG GGG CC
XmaI
Met-linker prptide-cGnRH-II portion based on ΔG value
ΔC C C C R S
HindIII
NedI
XmaI
XhoI
HindIII
PvuII

# FIG.9

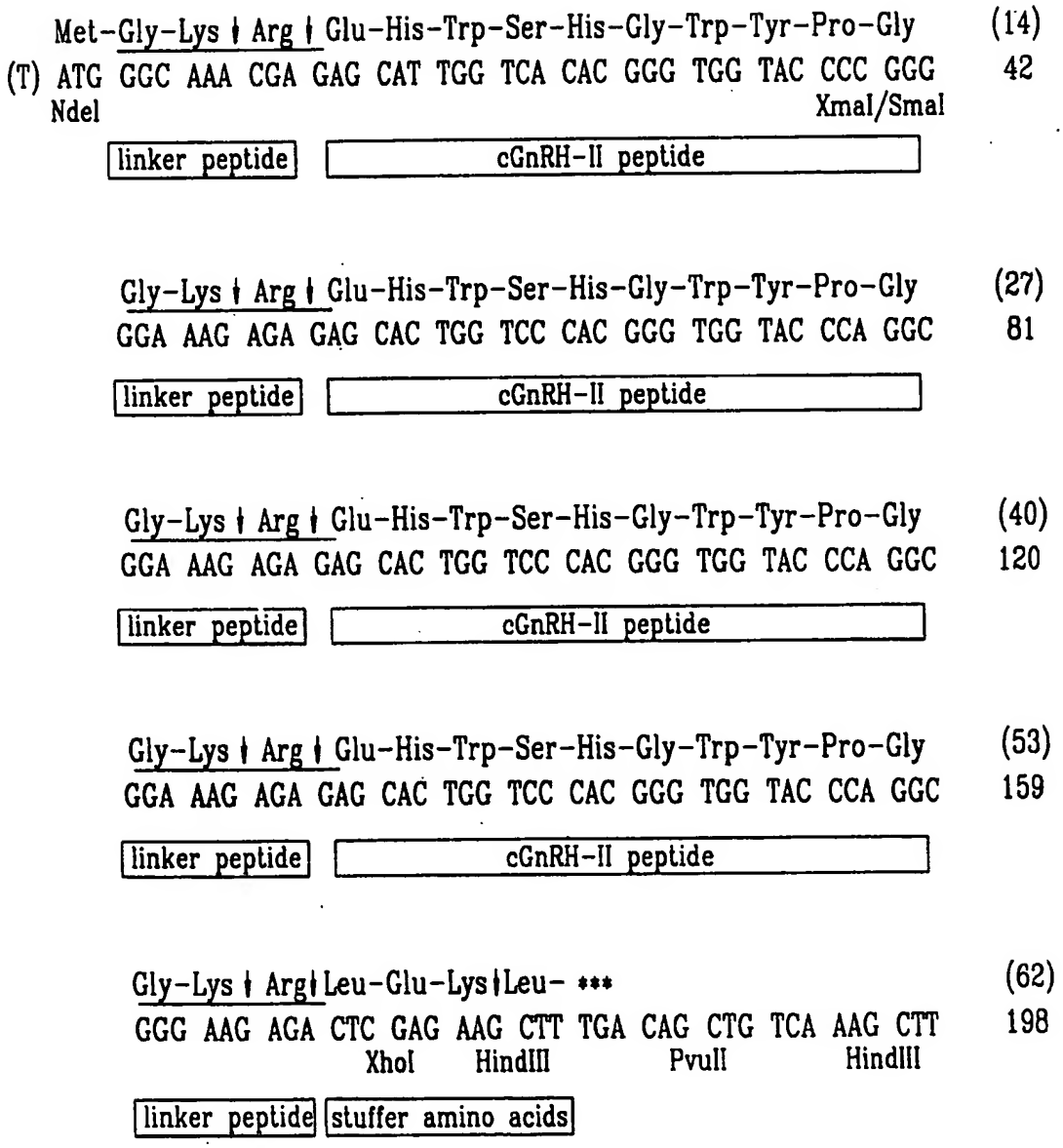

Met-Gly-Lys ↓ Arg ↓ Glu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly (14)
(T) ATG GGC AAA CGA GAG CAT TGG TCA CAC GGG TGG TAC CCC GGG 42
NdeI
XmaI/SmaI
linker peptide
cGnRH-II peptide
Gly-Lys ↓ Arg ↓ Glu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly (27)
GGA AAG AGA GAG CAC TGG TCC CAC GGG TGG TAC CCA GGC 81
linker peptide
cGnRH-II peptide
Gly-Lys ↓ Arg ↓ Glu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly (40)
GGA AAG AGA GAG CAC TGG TCC CAC GGG TGG TAC CCA GGC 120
linker peptide
cGnRH-II peptide
Gly-Lys ↓ Arg ↓ Glu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly (53)
GGA AAG AGA GAG CAC TGG TCC CAC GGG TGG TAC CCA GGC 159
linker peptide
cGnRH-II peptide
Gly-Lys ↓ Arg ↓ Leu-Glu-Lys ↓ Leu- *** (62)
GGG AAG AGA CTC GAG AAG CTT TGA CAG CTG TCA AAG CTT 198
XhoI
HindIII
PvuII
HindIII
linker peptide
stuffer amino acids

FIG. 10

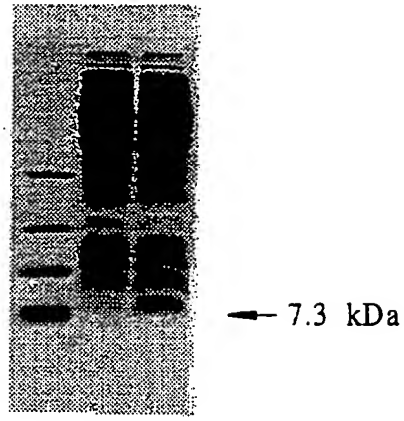
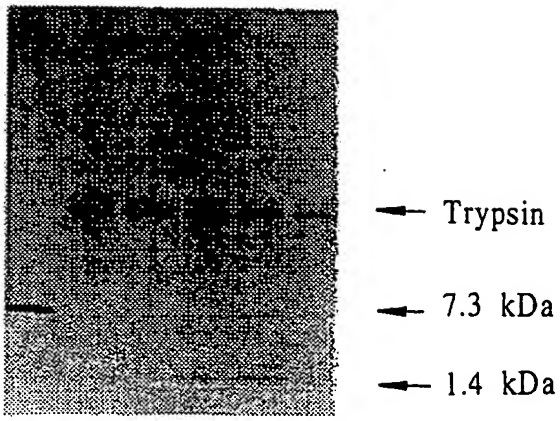



FIG. 11



# INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 99/00559

A. CLASSIFICATION OF SUBJECT MATTER

IPC[7]: C 12 N 15/00, 15/12, 15/62, 1/21 //(C 12 N 1/21; C 12 R 1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[7]: C 12 N 15/00, 15/12, 15/62, 1/21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
„A" document defining the general state of the art which is not considered to be of particular relevance
„E" earlier application or patent but published on or after the international filing date
„L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
„O" document referring to an oral disclosure, use, exhibition or other means
„P" document published prior to the international filing date but later than the priority date claimed
„T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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|---|---|
| 29 December 1999 (29.12.99) | 16 February 2000 (16.02.00) |
| Name and mailing adress of the ISA/AT<br>Austrian Patent Office<br>Kohlmarkt 8-10; A-1014 Vienna<br>Facsimile No. 1/53424/200 | Authorized officer<br>Mosser<br>Telephone No. 1/53424/437 |

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## INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 99/00559

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